MICROSPORIDIAN POLAR TUBE PROTEINS, NUCLEIC ACIDS CODING FOR THESE PROTEINS AND THEIR APPLICATIONS

RELATED APPLICATION

This is a continuation of International Application No. PCT/FR99/01630, with an international filing date of July 6, 1999, which is based on French Patent Application No. 98/08692, filed July 7, 1998.

FIELD OF THE INVENTION

This invention relates to purified complete microsporidian polar tube proteins (PTPs) as well as the genes coding these proteins and their use in the field of diagnosis.

BACKGROUND

E. cuniculi is a Microsporida, an obligate intracellular parasite, that occurs frequently in numerous mammals and is implicated in various infections in humans, principally in immunodepressed subjects. Two other species of the genus *Encephalitozoon* (*E. intestinalis* and *E. hellem*) are also implicated in various opportunistic infections. Generally speaking, the microsporidians are responsible in AIDS patients for gastrointestinal diseases, as well as ocular, muscular and hepatic disorders, rhinosinusitis and systemic infections [1]. Serological tests have also demonstrated the noteworthy presence of microsporidians in immunocompetent patients at the level of 8% of the population [2]. Four genera of microsporidians are responsible for human diseases: *Enterocytozoon*, *Encephalitozoon*, *Vittaforma* and *Trachipleistophora*. The emergence of these parasites in human pathology has created an increasing interest on the part of researchers in the systematic, epidemiological, clinical, diagnostic and therapeutic fields.

At present, diagnosis is based on PCR tests from oligonucleotides determined according to the ribosomal DNA sequences, the sole sequences known in most of the microsporidians. With regard to therapy, it is limited to certain compounds such as albendazole and fumagillin.

These unicellular eukaryotes exhibit a unique invasion mechanism. The spore, which is the infectious stage, contains an extrusion apparatus constituted by a polar tube inserted at its anterior end in an anchorage disk. Under the effect of certain stimuli, which can be linked *in vitro* to a variation in the pH, osmolarity, or the presence of cations or anions, the polar tube is extruded by the microsporidian spore and traverses the plasma membrane of a cellular host. The sporoplasm, expelled via this tube, is thereby inoculated into the receptor cell. These invasive apparatus, specific to the microsporidians and unique in the world of living organisms is, therefore, of great interest from not only the fundamental point of view but also from the applied point of view for diagnostics and therapeutics.

To date, no complete sequence of proteins constituting this polar tube has been obtained. According to Weidner [3], the polar tube is constituted by a single 23-kDa protein in *Ameson michaelis*. More recently, in a microsporidian parasite of fish, *Glugea americanus*, a differential extraction of the proteins in the presence of a reducing agent (DTT) made it possible to demonstrate that a 43-kDa protein is constitutive of the polar tube but only a part of the N-terminal sequence of 16 amino acids was determined [4]. Production of polyclonal and monoclonal antibodies against the polar tube of various species [5, 6, 7] has also been performed, thereby demonstrating a possible protein heterogeneity of this structure.

SUMMARY OF THE INVENTION

This invention discloses purified complete microsporidian polar tube proteins and the proteins, the amino acid sequences of which are represented in the attached sequence listings as SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4, SEQ ID No: 5. The invention discloses the genes coding these proteins and their use in the field of diagnosis.

BRIEF DESCRIPTION OF THE DRAWINGS

Characteristics and advantages of the invention will become manifest from the examples below concerning the production of antibodies against the polar tube, cloning and sequencing of the genes coding for the microsporidian polar tube proteins in *E. cuniculi* and which refer to the attached drawings in which:

Fig. 1 shows:

- A: electrophoretic separation of the sporal proteins;
- B: analysis by indirect immunofluorescence using polyclonal antibodies directed against the 55-kDa band separated by SDS-PAGE (1/50th dilution) on MRC-5 cells infested by *Encep[halitozoon cuniculi*; and
- C: immunoblot with the anti-55 kDa polyclonal antibodies (1/5000th dilution, track 1) and the monoclonal antibody Ec 102 (1/10,000th dilution, track 2).

Fig. 2 shows the immunoreactivity of the 55-kDa protein.

Fig. 3 illustrates the expression of PTP55 in *Escherichia coli*.

Fig. 4 shows an immunolabeling performed on two-dimensional electrophoresis gels with the monoclonal antibody directed against the polar tube.

Fig. 5 illustrates the expression of PTP35 in *Escherichia coli*.

DETAILED DESCRIPTION

The research studies that led to this invention consisted first of all of producing polyclonal and monoclonal antibodies against the polar tube of *E. cuniculi*. We thereby obtained two polyclonal antibodies (anti-55 kDa and anti-35 kDa) and one monoclonal antibody (anti-55 kDa) which react specifically with the polar tube in immunofluorescence and in electron microscopy [6]. After separation of the sporal proteins by two-dimensional electrophoresis and transfer onto PVDF membrane, a protein with an apparent molecular weight close to 55 kDa and an isoelectric point of 5 was recognized by these three types of antibodies. On these two-dimensional gels, another protein of apparent molecular weight close to 35 kDa and with an isoelectric point of 9 was also recognized by two anti-polar-tube antibodies, the polyclonal anti-35 kDa antibody and the monoclonal antibody.

The research studies performed in the context of this invention, therefore, for the first time made it possible to obtain the complete polar tube proteins of microsporidians. (The studies presented by the inventors at a congress (Fifth International Workshops on Opportunistic Protists and Fifth General Meeting of the European Concerted Action on *Pneumocystis* Research, Lille, September 3-7, 1997) on the production of a 55-kDa polar tube protein are insufficient to enable production of this complete and purified protein, and for the identification, cloning and sequencing of the corresponding gene. In fact, no nucleic or protein sequence data appear in the document recording this congress [8].)

The experimental protocol reported in that document comprises the conventional steps which are well known by those of ordinary skill in the art [9], such as extraction of the sporal proteins, electrophoreses (SDS-PAGE), production of polyclonal and monoclonal antibodies, microsequencing of peptides as well as the determination of degenerated primers and their amplification with PCR. In light of the synthetic character of the document recorded at the congress, its teaching is insufficient to allow one of

ordinary skill in the art to reproduce the inventors' work and produce a complete sequence of a complete microsporidian polar tube protein.

Thus, an object of the invention is the complete purified polar tube proteins of microsporidians and, more particularly, of three microsporidian species of the genus *Encephalitozoon*: *E. cuniculi*, *E. intestinalis* and *E. hellem*. More particularly, the invention pertains to:

- a protein of apparent molecular mass of approximately 55 kDa and an isoelectric point on the order of 5 from *E. cuniculi* and *E. intestinalis*,

- a protein of apparent molecular mass of approximately 35 kDa and an isoelectric point on the order of 9 from *E. cuniculi*, *E. intestinalis* and *E. hellem*.

In a second stage, the studies performed on the two purified proteins from *E. cuniculi* (55 and 35 kDa) comprised subjecting them to internal microsequencing after digestion with Endolysine C. Two peptides were sequenced in this manner (P1: ATALCSNAYGLTPGQQGMAC and P2: SATQYAMEACATPTP) for the 55-kDa protein and one peptide (P3: AVQGTDRCILAGIID) for the 35-kDa protein. It was possible using degenerated primers to amplify a part of the corresponding genes.

In the absence of a genomic library, we were able to determine the sequences of the genes and their flanking regions by means of an SSP-PCR technique [10]. As a result of these several stages of studies, it was possible to define the complete structure of the genes and their particularities as well as their possible similarities with other genes. The primary structures of the 55-kDa and 35-kDa proteins were also determined.

Thus, the invention pertains to the microsporidian polar tube proteins, the amino acid sequences of which are shown in the attached sequence listings as numbers SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4 and SEQ ID No: 5, a fragment or a functionally equivalent derivative thereof.

The phrase "functionally equivalent derivative" is understood to mean proteins, the sequences of which comprises a modification and/or a suppression and/or an addition of one or more amino acid residues, so long as this modification and/or suppression and/or addition does not substantially modify the function of these proteins. Such derivatives can be analyzed by one of ordinary skill in the art using the techniques of:

- screening expression libraries by means of antibodies directed against the polar tube proteins, or

- screening genomic libraries by means of nucleic probes capable of hybridizing with a gene coding for a polar tube protein.

The protein of 395 amino acids, referred to below as PTP55, represented in the attached sequence listing as SEQ ID No: 1 corresponds to the 55-kDa protein of *E. cuniculi*. It presents a deduced molecular weight of 36,609 Da and of 37,230 Da without the signal peptide, which is lower than that of 55,000 observed on polyacrylamide gels. This protein is synthesized by *E. cuniculi* in the form of a larger precursor the signal sequence of 22 amino acids of which is eliminated during screening towards the vesicles implicated in the formation of the polar tube. The sequence of a mature polar tube protein of the invention thus corresponds to the sequence comprised between the amino acids in positions 23 and 395 of SEQ ID No: 1. Various signal peptide characteristics can in fact be seen:

- predicted secondary structure forming an α helix,

- presence of hydrophobic amino acids as well as basic residues close to the N-terminal part,

- absence of a lysine residue in position 22, and

- prediction of signal peptide by von Heijne's algorithm [11].

In addition, the N-terminal sequencing of the protein demonstrated a sequence identical to that of the peptide P1, confirming that the peptide of 22 amino acids was cleaved upon maturation.

It can furthermore be seen that PTP55 does not contain tryptophan, phenylalanine or arginine residues. It presents a deduced isoelectric point of 4.7 in agreement with that seen on two-dimensional polyacrylamide gels which was on the order of 5. Investigation of these sequences of 395 amino acids and 373 amino acids in the mature protein shows that it does not present any significant homology with other proteins that have already been described in the databases.

A homologous protein of PTP55 was also identified in the species *E. intestinalis*. This protein of 371 amino acids is represented in the attached sequence listing as SEQ ID No: 3. The sequence presents notably strong homologies with the – and C- terminal regions of the PTP55 of *E. cuniculi*.

The protein of 277 amino acids, referred to below as PTP35, represented in the attached sequence listing as SEQ ID No: 2, corresponds to the 35-kDa protein of *E. cuniculi*. It presents a deduced molecular mass of 30,075 Da, thus smaller than that of 35,000 observed on polyacrylamide gels. The N-terminal end of PTP35 also presents signal peptide characteristics:

- predicted secondary structure forming an α helix,
- presence of hydrophobic amino acids as well as basic residues close to the N-terminal part,
- prediction of signal peptide by von Heijne's algorithm [11].

In the same manner as for PTP55, the PTP35 of *E. cuniculi* should present a signal peptide. Potential sites of proteolytic cleavage can be predicted between residues 12 and 13, 13 and 14 or 22 and 23. Nevertheless, such a cleavage could only be confirmed by

the sequencing of the N-terminal part of the protein. On the basis of the available data, the sequence of a polar tube protein according to the invention is comprised between amino acids 1 and 277 of the sequence presented in the attachment as SEQ ID No: 2.

PTP35 does not contain tryptophan residues. It presents a deduced isoelectric point of 8.6 in agreement with that observed on two-dimensional polyacrylamide gels which was on the order of 9. Investigation of this sequence of 277 amino acids demonstrates that it does not present any significant homology with other proteins already described in the databases.

We also obtained homologous proteins of PTP35 in two other species of the genus *Encephalitozoon*, *E. intestinalis* and *E. hellem*. The corresponding sequences are attached as SEQ ID No: 4 and SEQ ID No: 5. The PTP35 of *E. intestinalis* and of *E. hellem* are constituted of 275 and 272 amino acids, respectively, and present approximately 80% identity. The sequence of a PTP35 polar tube protein of the invention corresponds more particularly to a sequence constituted by or comprising:

- the sequence comprised between the amino acids in positions 1 and 275 of the sequence represented in the attached sequence listing as SEQ ID No: 4,

- the sequence comprised between the amino acids in positions 1 and 272 of the sequence represented in the attached sequence listing as SEQ ID No: 5.

Polyclonal or monoclonal antibodies directed against at least one protein of the invention or a fragment thereof can be prepared by the methods described in the literature. The polyclonal antibodies are formed according to the conventional techniques by injection of the proteins, extracted from the spores of *E. cuniculi* or produced by transformation of a host, to animals, then recovery of the antiserums and of the antibodies from the antiserums by, for example, affinity chromatography. The monoclonal antibodies can be produced by fusing myeloma cells with spleen cells from animals that have previously been

immunized with the proteins of the invention. These antibodies are useful for investigating other polar tube proteins of *E. cuniculi*, *E. hellem* or *E. intestinalis* and for studying the relationship between the polar tube proteins of different species or even different genera. In fact, the antibodies formed against the polar tube of *E. intestinalis* or *E. hellem* give rise to crossed immunological reactions with the polar tube proteins of *E. cuniculi*. But they can also find applications in the field of diagnostics.

The invention also pertains to a process for the diagnosis of infections caused by the microsporidians of the genus *Encephalitozoon* comprising the following steps:

a) a recombinant microsporidian polar tube protein according to the invention is immobilized on an analysis support such as a nitrocellulose film or an ELISA plate,

b) the aspecific reaction sites are saturated, for example, in the presence of 5% skimmed milk,

c) the product obtained in step (b) is incubated with the antibodies from the serum of the test subject in a manner such that if the serum contains antibodies directed against a microsporidian polar tube protein, they will complex with the protein,

d) the antibodies that did not complex in step (c) are eliminated from the serum by washing,

e) the product of step (d) is incubated with secondary antihuman antibodies coupled to a molecule enabling their visualization such as, for example, an enzyme such as peroxidase or a fluorochrome,

f) the antihuman antibodies that are not specifically bound are eliminated by washing, and

g) suitable means is used to visualize the antihuman antibodies / serum antibodies / protein complexes formed in step (e).

A diagnostic kit for the implementation of such a process is constituted by:

– an analysis support on which the recombinant microsporidian polar tube proteins are immobilized,

– a solution containing antihuman antibodies coupled to a molecule enabling their visualization, and

– instructions regarding the steps of the diagnostic process described above.

The invention also pertains to a nucleic acid molecule comprising or constituted by a nucleic sequence coding for a microsporidian polar tube protein. More particularly, the invention pertains to the nucleotide sequences coding for the proteins of PTP55 and PTP35 corresponding to the 55-kDa and 35-kDa proteins, respectively, of the microsporidians *E. cuniculi*, *E. intestinalis* and *E. hellem*.

The invention envisages specifically a nucleic acid molecule comprising or constituted by a nucleic sequence coding for a microsporidian polar tube protein the amino acid sequence of which is represented in the attached sequence listing as SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4 or SEQ ID No: 5, a fragment or a functionally equivalent derivative of this protein.

A DNA molecule comprising the sequence coding for the protein PTP55 of *E. cuniculi* is represented in the attached sequence listing as SEQ ID No: 1 or its complementary sequence. More particularly, such a nucleic acid sequence comprises the sequence comprised between the nucleotides 411 and 1532 of SEQ ID No: 1 or its complementary sequence. The nucleic sequence of SEQ ID No: 1 is composed of 1830 nucleotides and an open reading frame of 1188 base pairs going from position 345 (ATG initiation codon) to position 1532 (TAG stop codon). The region preceding position 345 is susceptible of comprising elements that are useful for the transcription of protein PTP55 such as a promoter region.

A DNA molecule comprising the sequence coding for a homologue protein of PTP55 identified in the species *E. intestinalis* is represented in the attached sequence listing as SEQ ID No: 3.

A DNA molecule comprising the sequence coding for protein PTP35 is represented in the attached sequence listing as SEQ ID No: 2 or its complementary sequence. More particularly, such a nucleic acid sequence comprises the sequence comprised between nucleotides 458 and 1291 of SEQ ID No: 2 or its complementary sequence. Nucleic sequence II of the invention is composed of 1740 nucleotides and comprises an open reading frame of 834 base pairs going from position 458 (ATG initiation codon) to position 1291 (TAA stop codon). The region preceding position 458 is susceptible of comprising elements that are useful for the transcription of protein PTP35 such as a promoter region.

Two DNA molecules each comprising a sequence coding for a homologue protein of PTP35 in two other species of the genus *Encephalitozoon*, *E. intestinalis* and *E. hellem* are represented in the attached sequence listing as SEQ ID No: 4 and SEQ ID No: 5.

The invention thus concerns, most particularly, the nucleic acid molecules the nucleotide sequences of which are represented in the attached sequence listing as SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4 and SEQ ID No: 5 as well as nucleotide sequences capable of hybridizing with these molecules. The invention also concerns the nucleotide sequences derived from SEQ ID No: 1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 4 and SEQ ID No: 5, for example, due to the degeneration of the genetic code, and which code for the proteins presenting the characteristics of microsporidian polar tube proteins.

The invention also pertains to a vector comprising at least one of the preceding nucleic acid molecules, advantageously associated with adapted control sequences, as well as a process for the production or expression in a cellular host of a microsporidian polar

tube protein of the invention or a fragment thereof. The preparation of these vectors as well as the production or the expression in a host of the proteins of the invention can be implemented by the molecular biology or genetic engineering techniques which are well known by the expert in the field.

As an example, a process for the production of a microsporidian polar tube protein according to the invention comprises:

- transferring a nucleic acid molecule of the invention or a vector containing said molecule into a cellular host,

- culturing said cellular host under conditions enabling production of the microsporidian polar tube protein,

- isolating said proteins by any appropriate means.

As an example, a process for the expression of a microsporidian polar tube protein according to the invention comprises:

- transferring a nucleic acid molecule of the invention or a vector containing said molecule into a cellular host,

- culturing said cellular host under conditions enabling expression of said proteins.

The cellular host employed in the preceding processes can be selected from among the prokaryotes or the eukaryotes, and especially from among the bacteria, yeasts, mammal cells, plant cells or insect cells. The vector employed is selected as a function of the host into which it will be transferred. It can be any vector such as a plasmid. The invention thus also pertains to the cellular hosts and, more particularly, to transformed bacteria such as *E. coli*, expressing the microsporidian polar tube proteins obtained in accordance with the preceding processes.

The invention also pertains to the nucleic probes and oligonucleotides prepared from the nucleic acid molecules of the invention. These probes, which are advantageously

labeled, are useful for the detection by hybridization of similar sequences in other microsporidians. By means of the conventional techniques, these probes are brought into contact with a biological sample. Various hybridization techniques can be employed such as hybridization on blots (Dot-blot) or hybridization on replicas (Southern technique) or other techniques (DNA chips). Such probes constitute tools enabling rapid detection of similar sequences in the genes coding for the microsporidian polar tube proteins which makes it possible to study the origin and the conservation of these proteins constituting the polar tube.

The oligonucleotides are useful for PCR experiments, for example, for investigating the genes in other microsporidians or for diagnostic purposes.

The invention thus also pertains to a process for the diagnosis of infections caused by microsporidians, comprising the following steps:

a) the DNA is extracted from microsporidian spores taken from biological samples of urine, stool or a biopsy,

b) the extracted DNA is amplified by any suitable means such as PCR with specific oligonucleotides deduced from the sequences of the genes coding for the microsporidian polar tube proteins,

c) the amplification products are immobilized on an analysis support,

d) the microsporidian origin of the amplification products is determined by hybridization by means of a labeled nucleotide probe specific to a microsporidian.

It is possible to perform step (c) by fixation of the amplification products on an analysis support such as a membrane or an ELISA plate.

A diagnostic kit for the implementation of such a process is constituted by:

- the means required for the amplification of the sequences coding for the microsporidian polar tube proteins, such as the specific oligonucleotides of these sequences

and all other elements necessary for the performance of a PCR,

- an analysis support for fixing the amplification products, and

- labeled probes specific to a microsporidian.

The invention also pertains to vaccinal compositions which prevent infections caused by the microsporidians of the genus *Encephalitozoon* comprising as active principle a protein of the invention or a fragment of the protein in association with a pharmaceutically acceptable vehicle. In fact, since the antibodies formed against the polar tube of *E. intestinalis* or *E. hellem* cause crossed immunological reactions with the polar tube proteins of *E. cuniculi*, the invention advantageously provides a potential vaccine against the infections caused by the microsporidians of the genus *Encephalitozoon*.

Turning now to the drawings, Fig. 1 shows:

- A: electrophoretic separation of the sporal proteins by SDS-PAGE with on track 1 the soluble fraction in 2% SDS, 10% 2-mercaptoethanol, and on track 2 the residual fraction obtained after incubation in 50% 2-mercaptoethanol for 48 hours.

- B: analysis by indirect immunofluorescence using polyclonal antibodies directed against the 55-kDa band separated by SDS-PAGE (1/50th dilution) on MRC-5 cells infested by *Encephalitozoon cuniculi*. The spores with their extruded polar tubes are strongly marked.

- C: immunoblot with the anti-55 kDa polyclonal antibodies (1/5000th dilution, track 1) and the monoclonal antibody Ec 102 (1/10,000th dilution, track 2). The molecular weight markers (M) are indicated on the left and given in kDa. The blots were visualized using an ECL kit (Amersham).

Fig. 2 shows the immunoreactivity of the 55-kDa protein. Two-dimensional gel electrophoresis was performed using isoelectrofocalization in the first dimension and 12% gels in the second dimension. The separated proteins were either stained with silver nitrate

(A) or transferred onto PVDF membranes and incubated with the polyclonal antibodies directed against the 55-kDa acid spot (1/5000th dilution) isolated by 2D electrophoresis (B). The molecular weights are indicated in kDa and the isoelectric points are numbered from 4 to 8. The specific labeling of the extruded polar tubes in immunofluorescence with this antibody can be seen in (C).

Fig. 3 illustrates the expression of PTP55 in *Escherichia coli*. A shows the analysis on polyacrylamide gels (SDS-PAGE) of the proteins extracted from bacteria transformed with the plasmidic construction pQE30-PTP55. Track 1 shows production without induction, track 2 shows the state after induction with IPTG and track 3 shows the recombinant PTP purified on Ni-NTA resin.

B shows an immunoblotting with the serums directed against the recombinant PTP55 (1/1000th dilution). On track 1, the *E. coli* proteins 4 hours after IPTG induction; on track 2, the proteins from *Encephalitozoon cuniculi*.

C shows a labeling with indirect immunofluorescence with the antiserums directed against the recombinant PTP55 of the polar tubes of *E. cuniculi*. The extruded polar tubes are indicated by arrows.

D shows an immunolabeling with colloidal gold in transmission electron microscopy of the polar tube sections.

Fig. 4 shows an immunolabeling performed on two-dimensional electrophoresis gels with the monoclonal antibody directed against the polar tube.

Two-dimensional gel electrophoresis was performed using isoelectrofocalization in the first dimension and 12% gels in the second dimension. The separated proteins were either stained with silver nitrate (A) or transferred onto PVDF membranes and incubated with the monoclonal antibodies (1/5000th dilution) (B). The 55-kDa and 35-kDa spots are indicated by arrows.

Fig. 5 illustrates the expression of PTP35 in *Escherichia coli*. Fig. 5 shows:

in A, polyacrylamide gel analysis (SDS-PAGE) of the proteins extracted from bacteria transformed with the plasmidic construction pQE30-PTP35. Track 1 shows production without induction, track 2 shows the state after induction with IPTG and track 3 shows the recombinant PTP purified on Ni-NTA resin. The molecular weight markers are indicated in kDa.

- in B, an immunoblotting with the serums directed against the recombinant PTP35 ($1/1000^{th}$ dilution).

Track 1 shows the electrophoretic profile of *Encephalitozoon cuniculi* stained with Coomassie blue.

Track 2 illustrates the labeling of a 35-kDa protein from *E. cuniculi* with the antibody directed against the 35-kDa recombinant protein expressed in *Escherichia coli*.

- in C, the indirect immunofluorescence labeling with the antiserums directed against the recombinant PTP35 of the polar tubes of *E. cuniculi*. The arrow indicates an extruded polar tube.

1) Production of antibodies against the polar tube of *E. cuniculi*, immunocytochemical analyses

The strain of *E. cuniculi* employed was a mouse isolate. It was maintained in MDCK cellular culture. The spores released in the culture supernatant were recovered and stored at 4°C in PBS. Extraction of the sporal proteins was performed by grinding the spores with zirconium balls (0.1 mm in diameter) in a buffer containing 2.5% SDS and 10% 2-mercaptoethanol in the presence of protease inhibitors. After heat denaturation for 10 minutes at 100°C, the sporal debris was eliminated by centrifugation at 18,000 g for 5 minutes. The proteins were then separated by SDS-PAGE on 12% polyacrylamide gels.

For the two-dimensional electrophoresis, the protein samples were solubilized in a buffer based on 9 M urea, 5% 2-mercaptoethanol and 40 mM CHAPS. Isoelectro-ocalization was performed under the following conditions: 4 hours at 400 V, 30 minutes at 600 V then 30 minutes at 800 V with the combination of 40% pH 3-10, 60% 4-6.5 ampholines (Pharmacia). After equilibration of the first-dimension gels in SDS / 2-mercaptoethanol for 10 minutes, the proteins were separated according to their molecular mass by SDS-PAGE. The corresponding gels were dyed with either silver or Coomassie blue, or transferred onto PVDF membrane (Immobilon P, Polylabo) using a semi-dry system.

Polyclonal antibodies were produced against various *E. cuniculi* proteins separated by electrophoresis. Intraperitoneal injections were performed in BALB/c mice for each protein sample. The 55-kDa protein band was also used to produce monoclonal antibodies. Thus, three antibodies directed against the polar tube were obtained: two anti-35-kDa and anti-55-kDa polyclonal antibodies and one anti-55-kDa monoclonal antibody.

Immunoblotting, immunolocalization in IFA and in transmission electron microscopy were performed using conventional techniques.

2) Microsequencing PTPs of apparent molecular masses 55 kDa and 35 kDa

The N-terminal sequence as well as two internal peptides (P1 and P2) were sequenced for the PTP55 of *E. cuniculi*.

N-terminal: ATALCSNAYG

P1: ATALCSNAYGLTPGQQGMAQ

P2: SATQYAMEACATPTP

One internal peptide (P3) was sequenced for the PTP35.

P3: AVQGTDRCILAGIID

These sequences were performed on the 55-kDa and 35-kDa proteins isolated by two-dimensional electrophoresis, by the Protein Microsequencing Laboratory, Institut Pasteur, Biotechnology Department.

For the internal sequencing of the peptides P1, P2 and P3, the proteins were first digested by Endolysine C, proteolytic enzyme cutting after a lysine residue.

3) PCR amplification, cloning and sequencing of the genes coding for the PTPs

a) Genes coding for the PTP55 of *E. cuniculi* and *E. intestinalis*

From degenerated primers deduced from the peptides P1 and P2, a DNA fragment of approximately 1 kpb was amplified, cloned in a plasmidic vector pCR2 (Invitrogen, TA cloning vector) and sequenced according to Sanger's method [12]. Amplification of the 5' and 3' regions of the gene of the PTP was performed by a PCR technique (SSP-PCR). Analysis of the sequences was performed on the molecular biology server Infobiogen.

The complete sequence represented in the attached sequence listing as SEQ ID No: 1 comprises 1830 nucleotides and has a reading frame of 1188 pb. This frame contains 395 codons going from the site considered to be the translation initiation site to the TAG termination codon. The codon considered to be the ATG departure codon is preceded by a region that is particularly rich in A-T. The translated amino acid sequence is represented in the attached sequence listing as SEQ ID No: 1.

By means of PCR and SSP-PCR amplifications, the gene coding for a homologous protein of PTP55 was sequenced and is represented in the attached sequence listing as SEQ ID No: 3. This sequence comprises a reading frame of 1113 pb. This frame contains 371 codons going from the site considered to be the translation initiation site to the TAG termination codon.

b) Genes coding for the PTP35 of *E. cuniculi*, *E. intestinalis* and *E. hellem*

From degenerated primers deduced from the peptide P3, different fragments were amplified by the SSP-PCR technique, cloned in a plasmidic vector pGEMT (Promega, TA cloning vector), sequenced according to Sanger's method and analyzed as described above.

The complete sequence of the PTP35 of *E. cuniculi* represented in the attached sequence listing as SEQ ID No: 2 comprises 1740 nucleotides. The reading frame comprises 834 pb. This frame contains 277 codons going from the site considered to be the translation initiation site to the TAA termination codon. The codon considered to be the ATG departure codon is preceded by a region that is particularly rich in A-T, similar to that of PTP55. The translated amino acid sequence is represented in the attached sequence listing as SEQ ID No: 2.

The sequences of the PTP35 of *E. intestinalis* and *E. hellem* represented in the attached sequence listing as SEQ ID No: 4 and SEQ ID No: 5 contain, respectively, 825 and 816 nucleotides not including the stop codon. The corresponding proteins are constituted by 277 and 272 amino acids.

4) Expression of the PTPs in *Escherichia coli*

A part of the PTP55 of *E. cuniculi* corresponding to the region between the peptides P1 and P2 was cloned in an expression vector pQE30 (Qiagen) and expressed in *E. coli* (strain M15). The recombinant protein was purified by affinity chromatography on nickel columns and injected in mice. The corresponding antibodies tested in immunoblotting, immunofluorescence and transmission electron microscopy made it possible to confirm that this protein was in fact localized at the level of the *E. cuniculi* polar tube.

A part of the PTP35 between the residues 27 and 277 was also expressed in *E. coli* using the same technique. The antibodies produced against this recombinant protein exhibited a labeling of the polar tube.

5) Analysis of the primary sequences of the PTP55 and PTP35

Blast analysis did not reveal any significant homology with other known proteins with the exception of collagen, principally due to the fact that PTP55 is rich in glycine and proline residues.

a) The PTP55 are rich in proline, glycine, glutamine, serine and threonine residues with these five accounting for more than 55% of the amino acid content. The proposed cleavage site (between the serine and alanine residues of the PTP55 of *E. cuniculi*) is predicted as such by the following characteristics:

- absence of lysine residue in position 22 preceding the P1 peptide (23-42) sequenced after digestion of the protein with Endolysine C,
- N-terminal sequencing of the protein corresponding to that of the peptide P1,
- presence of hydrophobic amino acids in this N-terminal region,
- von Heijne's algorithm,
- secondary structure in α helix.

The PTP is most likely synthesized by *E. cuniculi* (or *E. intestinalis*) in the form of a larger precursor the 22-amino-acid signal sequence of which is eliminated upon maturation. The mature protein would, therefore, have a molecular mass of 37,230 Da.

N-glycosylation sites (NETS, NGTS and NISG) are present in the sequence. The presence of numerous serine and threonine residues (21.6%) is also suggestive of O-glycosylation sites.

The central region of the protein PTP55 of *E. cuniculi* is characterized by four repetitions in tandem of 26 amino acids, each with a conservation at the nucleic level. This region is partially framed by two other repetitions of 9 amino acids. No repetition was seen in the PTP55 sequence of *E. intestinalis*, but the two PTP55 present strong homologies in the N-terminal and C-terminal parts.

b) The PTP35 are particularly rich in lysine residues (11.5%) and glutamic acid (9%). Three potential cleavage sites of a signal sequence are represented between the residues 12 and 13, 13 and 14, and 22 and 23. An RGD sequence is present in the PTP35 of *E. cuniculi* and *E. intestinalis*; this sequence is also found in proteins such as fibronectin and intervenes in cellular attachment phenomena. A potential N-glycosylation site (NSTS) is also present in the PTP35 sequence of *E. cuniculi*.

6) Chromosomal localization and estimation of the number of copies

Hybridization of a probe corresponding to a part of the gene coding for PTP55 on the chromosomes of *E. cuniculi* separated by pulsed field electrophoresis revealed a unique localization of this gene on chromosome VI.

The same probe was applied on Southern blots after digestion of the genomic DNA of *E. cuniculi* by different restriction enzymes: a single band is marked on each digestion profile which makes it possible to affirm that the gene exists in a single copy.

The gene coding for the PTP35 of *E. cuniculi* is also localized on chromosome VI.

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